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# PATENT SPECIFICATION

NO DRAWINGS

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COMPLETE SPECIFICATION

## A process for the Modification of Proteins

We, BIOTEST-SERUM-INSTITUT G.M.B.H., a German Body Corporate of 4, Flughafenstrasse, Frankfurt/Main, Germany, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to a method of producing modified protein preparations for use more particularly as blood plasma substitutes.

In cases of heavy bleeding or shock, for example after accidents, it is very important to replenish the patient's circulation by the supply of solutions having the same osmotic pressure as blood plasma. Solutions of gum arabic or non-modified gelatin were originally used for this purpose, but they have a number of undesirable side effects. The introduction of polyvinyl pyrrolidone and dextran preparations was an advance, but no decisive advantages were obtained until use was made of modified gelatin solutions whose physical properties had been modified by chemical reactions.

All these modifications were provided in order to give blood plasma substitutes adapted to satisfy the many requirements made of them. For these products to be of use clinically, they must also maintain the osmotic pressure in addition to simply replenishing the circulation. With regard to the physical properties of the products, their molecular weights must be high enough to guarantee adequate retention time in the circulation, and the preparations have to be liquid down to low temperatures, stable over a wide temperature range and during storage, and readily sterilisable. The substitute must be neither pyrogenic nor antigenic and must be neither acutely nor chronically toxic to the tissues, i.e., it should be separated or assimilated without producing any organic disturbances and should not be deposited. In addition, reproducible and inexpensive manufacture of the preparations must be possible in any quantities. In the attempt to satisfy these requirements in prior methods, gelatin in the form of aqueous solutions was decomposed either by controlled heating or by chemical or enzymatic treatment, and was then re-condensed with aldehydes or polycarboxylic acid anhydrides or the corresponding chlorides. Methods are also known wherein the gelatin is first cross-linked with aldehydes, dialdehydes, diisocyanates, quinones, and the like, and then partially oxidised with hydrogen peroxide. The attempt has also been made to prepare suitable products by blocking the gelatin molecules or the reaction products thereof at the amino groups, thus lowering the iso-electric point and raising the osmotic pressure.

These processes have a number of disadvantages. For example, in some of the steps the reaction was unspecific and hence difficult to control and reproduce. The resultant solutions were also very heterogeneous and in some cases the solutions used were contra-indicated for certain disorders, for example nephrotic illnesses. Chemicals which are incorporated as bridge links were used practically without exception and the excess reagent had to be destroyed or separated from the protein by precipitation and reprecipitation. It is necessary, therefore, to overcome the above disadvantages and satisfy requirements by so controlling the cross-linking of the proteins, for example the filamentary gelatin molecules, so that new cross-linking peptide bonds are formed between the

filamentary molecules without the addition of bridging links, and then decomposing the resultant macromolecule at the original peptide linkages by enzymatic or chemical means to the required extent. In this way the highly viscous and readily gelatinising filamentary molecules give spherical molecules of low viscosity but relatively high molecular weight their aqueous solutions remaining liquid over a wide temperature range.

To this end, according to the invention, free carboxyl groups of the protein molecule are first reacted in manner known *per se* with the amino groups thereof, *i.e.*, directly by forming new cross-linking peptide linkages, without the interposition of other groups, and then preferentially some of the original peptide bonds of the protein molecule are split. In a preferred procedure, the free carboxyl groups of the protein are caused to react with the amino groups thereof by activation in manner known *per se*, or conversely, the amino groups are activated to bring about the same reaction.

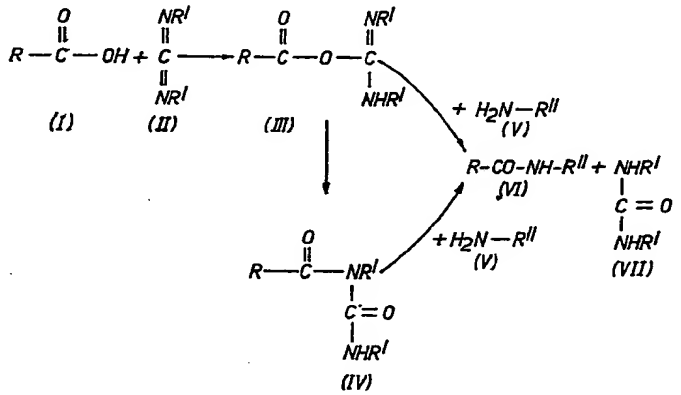
The free carboxyl groups of the protein can be activated with carbodiimidines, preferably with N,N$^1$-disubstituted carbodiimides and with nitrogen analogues of ketenes. Dicyclohexyl and cyclohexylmorpholinyl ethyl carbodiimide may be given as examples of carbodiimides.

The free carboxyl groups, *i.e.* including the lateral carboxyl groups of glutamic and aspartic acid may be activated similarly to the Sheehen and Hess method known in peptide synthesis (Sheehen, J. C., Hess, G. F., Journal of the American Chemical Society *77*, 1067 (1955)).

The activated carboxyl groups can thereupon be reacted under mild conditions with primary amino groups in the protein molecules for example with the N-terminal $\alpha$- and the lateral $\epsilon$-amino groups of lysine and hydroxylysine.

In contrast to most other methods which can be used for activation of the carboxyl or amino group or amino acids, the reaction with carbodiimide takes place at room temperature or slightly elevated temperature. It is relatively insensitive to moisture, so that it can also be carried out in aqueous solution. It is also very selective, so that acyl hydroxyamino acids and acyl arginine can be reacted without an additional protective group and there is no danger of any appreciable racemisation.

The reaction is probably as shown by the following diagram:




N,N$^1$-disubstituted carbodiimide (II) is added to the carboxyl component (I) with the formation of O-acyl-isourea (III), which is readily transposed by acyl migration to a N-acyl-ureide (IV), which reacts with an amino component (V) to form the acid amide or peptide (VI) and N,N$^1$-disubstituted urea (VII). The addition of the amino component (V) to the O-acyl-isourea (III) with the formation of (VI) and (VII) is a further possibility.

The urea derivative formed from carbodiimide is usually difficultly soluble or insoluble so that it can be removed by ordinary filtration.

The resultant product is then decomposed by preferential splitting of some of the original peptide bonds of the protein molecule. The decomposition may be carried out enzymatically for example. It can also be carried out by the action of heat or chemically or by a combination of both steps. The chemical decomposition may be achieved by means of an acid hydrolysis.

Thus in one exemplified embodiment of the method according to the invention, a 5—10% gelatin solution or some other suitable protein solution e.g. globin de-

composition products, albumen or globulin decomposition products or casein decomposition products — which may already be partially decomposed — may be reacted at a pH of from 3 to 6 and at from 40 to 80°C with an N,N¹-disubstituted carbodiimide to form a highly viscous cross-linked product and then split at a pH of from 7 to 8 and at from 30 to 40°C by treatment with a tryptic enzyme which is known to split peptide linkages specifically at the carboxyl side of arginine and lysine residues only provided the basic groups of the side chains of these residues are in a free state. The degree of decomposition can be varied as desired by appropriate selection of the incubation time and the type and quantity of enzyme and the enzymatic action can be stopped by the addition of trasylol, a physiological padutin inhibitor. After filtration to remove the precipitated urea derivative and sterilisation the solutions are ready for use. Depending on the purpose for which the product is intended, the reaction may be carried out in water or physiological common salt solution or else the peptides of low molecular weight can be separated by dialysis or therapeutically valuable substances may be added before sterilisation.

The method according to the invention has the advantage that inter- and intramolecular cross-linking peptide bonds are formed between the free carboxyl groups and amino groups in the protein molecules, preferably gelatin, under mild conditions, by completely specific activation, and this, *inter alia*, distinguishes it from all previously known methods. The modified protein preparations obtained according to the invention contain no foreign linking elements of any kind and therefore have very favourable physiological properties. The method according to the invention also has the advantage that the decomposition of the cross-linked products achieved as well by specific enzymatic as by less specific chemical means, e.g. by partial acid hydrolysis gives spherical molecules of low viscosity but relatively high molecular weight, the solutions of such molecules being liquid over a wide temperature range and having a sufficiently long retention time in the circulatory system.

Another advantage of the method is that the ratio of the proportions of high molecular weight to those of low molecular weight in the reaction product can be varied by appropriate selection of the reaction conditions during cross-linking and decomposition. The method also has the advantage that apart from the activation reagent which is removed at the end of the reaction no other chemicals are used and which makes it necessary to purify the reacted proteins by precipitation and reprecipitation. The reaction product of the activation substance can readily be removed, for example by filtration, owing to its difficulty soluble nature.

Another advantage of the method is that the reaction can be carried out in water or physiological common salt solution depending upon the required purpose e.g. when converting long-active products into spherical products of similar molecular weight and the addition of therapeutically valuable substances does not impair the stability of the solutions.

The following examples illustrate the invention:

## EXAMPLE I

1 litre of a 7.5% gelatine solution in 0.9% sodium chloride is adjusted to pH 4.5. A solution of 7.5 g of N,N¹-dicyclohexyl carbodiimide in 100 c.c. of absolute ethanol is added over a period of from 5 to 10 minutes at 60°C, with agitation.

To obtain a homogeneous highly viscous reaction product, 350 c.c. of 0.9% sodium chloride solution is added for dilution over the next 30 to 40 minutes.

After one hour, counting from the beginning of the reaction, the solution is cooled to 37°C, the pH is brought up to 7.5 with N—NaOH solution, and 5 mg of trypsin dissolved in 10 c.c. of physiological common salt solution with the addition of a few drops of N—HCl, are added to the solution. After another 20 minutes the enzymatic decomposition is stopped by the addition of 10 c.c. of trasylol and the solution is made isotonic by the addition of 1.4 g of solid sodium chloride and possibly other salts. The protein concentration is 5%. It is separated from the precipitated urea derivative by filtration and sterilised at 120°C.

## EXAMPLE 2

800 c.c. of water are added to 50 g of gelatin having an iso-electric point of about pH 5, the mixture is allowed to swell and the gelatin to dissolve at 40°C. 5 g of N,N¹-dicyclohexylcarbodiimide dissolved in 50 c.c. of absolute ethanol are added in drops with agitation and agitation is continued for another hour.

The pH is adjusted to from 7 to 8 and the mixture is decomposed for 20 minutes

with trypsin. The reaction is stopped by the addition of trasylol. The urea derivative is filtered off and the filtrate is made up to 1 litre with water. The solution is made isotonic by the addition of sodium chloride and sterilised.

EXAMPLE 3

60 g of gelatin having an isoelectric point of about pH 5 is allowed to swell in 800 c.c. of water and is dissolved at 40°C. The pH value of the solution is set to 7.5. The gelatin is decomposed for ten minutes with 4 mg of trypsin. The pH value is then adjusted to 4.5 and the solution is cross-linked with 6 g of carbodiimide dissolved in 80 c.c. of absolute ethanol, with agitation.

After three hours the pH is re-adjusted to 7.5 and the reaction mixture decomposed for 10 minutes with 4 mg of trypsin. After the addition of 8 c.c. of trasylol the solution is filtered, made up to one litre, made isotonic, and sterilised.

EXAMPLE 4

40 g of ion-exchanged bone gelatin is allowed to swell in 600 c.c. of sterilised water with agitation at from 50 to 60°C, dissolved, and brought to pH 4 with N—HCl. At a bath temperature of 100° and with agitation, heating is continued until the viscosity has dropped by about one-third. The temperature is then reduced to from 70 to 75°C and a solution of 2 g of dicyclohexyl carbodiimide in 100 c.c. of non-denatured ethanol is added within a period of 20—30 minutes with agtiation and agitation is continued until the viscosity is approximately doubled. The temperature is then again brought to 100°C and decomposition continues until the viscosity has again reached the value of the original solution.

The mixture is filtered from the majority of the used activator, 9 g of NaCl are added to the solution the pH is adjusted to 7.2—7.4 with N—NaOH solution and made up to 1 litre.

The finished solution is filtered and packed under sterile conditions and sterilised for 30 minutes at 120°C.

WHAT WE CLAIM IS:—

1. A method of producing modified proteins particularly for use as blood plasma substitutes, wherein free carboxyl groups of the protein molecule are first reacted by forming inter- and intramolecular cross-linking peptide linkages with the amino groups thereof and then preferentially some of the original peptide bonds of the protein molecule are split.

2. A method as claimed in claim 1, wherein the free carboxyl groups of the protein are reacted with the amino groups thereof by means of activating the functional groups.

3. A method as claimed in claim 1 or claim 2, wherein the free carboxyl groups of the protein are activated by treatment with carbodiimides, preferably $N,N^1$-disubstituted carbodiimides.

4. A method as claimed in claim 1 or claim 2, wherein the original peptide bonds are split enzymatically.

5. A method as claimed in claim 1 or claim 2, wherein the original peptide bonds are split by the action of heat and/or chemical decomposition, more particularly acid hydrolysis.

6. A method as claimed in any of the preceding claims, wherein gelatin is used as protein.

7. A method of producing modified proteins substantially as described herein with reference to the foregoing examples.

8. Modified proteins whenever prepared by the process of claims 1 to 7.

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